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Effects of missense mutations on rat aquaporin-2 in LLC-PK1 porcine kidney cells

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Background. Mutations in the aquaporin-2 (AQP2) gene have been found in families with nephrogenic diabetes insipidus (NDI), but the pathophysiological mechanisms of how mutant AQP2 causes the disease are still not clear.

Methods. Wild-type (WT) AQP2 and four mutants—T126M, A147T, R187C, and S216P—were transiently expressed in LLC-PK1 cells. The osmotic water permeability of LLC-PK1 cells expressing AQP2 mutants was determined by stopped-flow light-scattering microphotometry. Cell surface expression, subcellular localization, and effects of vasopressin stimulation were examined by surface biotin labeling and confocal immunohistochemistry.

Results. The osmotic water permeability (Pf) of cells expressing WT increased significantly after vasopressin treatment, whereas the Pf of cells expressing T126M A147T, R187C, and S216P was not significantly different from that of the control even after vasopressin stimulation. Confocal immunohistochemistry demonstrated distribution of WT and A147T in early/recycling endosomal compartments and vasopressin-responsive translocation and surface expression. In contrast, stainings of T126M, R187C, and S216P were similar to that of Grp78, indicating that these mutants were misassembled and retarded in the endoplasmic reticulum.

Conclusion. Our results indicated that the intracellular distribution and vasopressin-regulated trafficking of A147T is intact, in contrast to the other three mutants, of which both were impaired. Thus, it is conceivable that the disruption of the AQP2 channel function accounts for the pathogenesis of A147T NDI, whereas trafficking defects account for that of the other types, suggesting that the pathophysiology of AQP2-related NDI is heterogeneous.

Aquaporin-2 (AQP2) is the vasopressin-regulated water channel exclusively expressed in kidney collecting duct of mammals [1–5]. The importance of AQP2 for water and electrolyte homeostasis has been proven by the finding that mutations in the AQP2 gene cause nephrogenic diabetes insipidus (NDI) [5–10]. Although more than 20 mutations in the AQP2 gene that cause inherited NDI have been reported, molecular mechanisms of the pathogenesis of NDI related to AQP2 mutations have been controversial and unclear [5–12].

Severe defects in trafficking of the AQP2 mutants T126M, A147T, R187C, and S216P have been reported by various authors. In a series of work that used *Xenopus* oocytes as a heterologous expression system [5–8], AQP2 mutants could be barely detected on the plasma membrane. However, Goji et al showed that two NDI mutants, T125M and G175R, have no apparent defect in their routing to the plasma membrane but have a disruption in water channel function [10], suggesting that nonfunctional channels may take part in the pathology of NDI. In work done by Tamarappoo and Verkman, water permeabilities of T126M and A147T were suggested to be comparable to that of the wild type (WT), based on minimal increases in oocyte water permeability and semiquantitative estimation of surface expression by immunoprecipitation [11]. These discrepancies raise questions about the functional and structural properties of amino acids near the fourth transmembrane domain and in the fifth hydrophilic loop, where a majority of the AQP2 mutants exist.

Although analysis of intracellular distribution and vasopressin-regulated trafficking of AQP2 mutants in epithelial cells have been needed to overcome limitations of observations with nonepithelial cells such as oocytes, only a few studies have been reported using mammalian epithelial cells [11, 13–16]. Mutants R187C, T126M, and A147T were shown to have trafficking defects in CHO and MDCK cells [11]; however, vasopressin-related translocation of these mutants has not been examined in vasopressin-responsive epithelial cells such as LLC-PK1 and

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Madin-Darby canine kidney (MDCK) cells, as defects in channel regulation may also be one of the features of NDI.

To examine the effects of mutations on AQP2 function, intracellular localization, and regulated trafficking, WT and a series of AQP2 mutants were transiently expressed in LLC-PK1 cells, a cell type that has been demonstrated to be useful as a practical model for the study of regulatory trafficking of AQP2 [13–15]. We avoided tagging AQP2, as it may affect function and trafficking of AQP2. The subcellular distribution of AQP2 and its mutants could be precisely visualized by using rat AQP2 cDNA and affinity-purified antibody.

METHODS

Site-directed mutagenesis

Missense mutants of rat and human AQP2, T126M, A147T, R187C, and S216P were constructed by polymerase chain reaction using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) (Fig. 1). These four mutants were selected because the characteristics of these mutants have been well demonstrated in patients and other cell expression systems. The nucleotide sequences of both strands were verified by a fluorescence sequencer (ABI 337; Applied Biophysics, Foster City, CA). WT and mutant AQP2 were subcloned into the HindIII, XbaI site of the mammalian cell expression vector pcDNA3 (Invitrogen, San Diego, CA, USA).

Transient cell expression

LLC-PK1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 50 U/ml penicillin G and 50 μg/ml streptomycin until semiconfluency on a 15 cm dish. The cells were treated with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA), were suspended in 600 μl K-PBS (30.8 mM NaCl, 120.7 mM KCl, 81 mM Na_2HPO_4, 5.0 mM $MgCl_2$) (approximately 1×10^7/ml), and were transfected with 20 μg of WT or mutant AQP2 DNA by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA, USA) at 360 mV, 960 μF. After incubation at room temperature for 10 minutes, cells were plated and cultured for approximately 24 hours at 37°C [15]. Although Western blots detected comparable expression of rat and human AQP2, immunofluorescence showed faint staining of human AQP2 (data not shown) in contrast to vivid staining of rat AQP2. Thus, further mutational analyses were performed using rat AQP2 mutants. Differences in immunodetection between Western blots and immunohistochemistry are possibly due to effects of fixation [17].

Western blotting

After being cultured for approximately 24 hours, transfected cells were lyzed in Reporter Lysis Buffer (Promega, Madison, WI, USA) at room temperature for 15 minutes. After centrifugation at 1500 × *g* for five minutes, the whole cell lysates were suspended in sodium dodecyl sulfate (SDS) sample buffer (Owl Scientific, Woburn, MA, USA), denatured at 70°C for 10 minutes, separated in 10 to 20% gradient polyacrylamide gel at 40 mA for one hour, and transferred to Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) at 140 mA for one hour with a semidry transferring system (Biometra, Goettingen, Germany). After blocking the membrane overnight in SuperBlock in TBS (Pierce, Rockford, IL, USA), it was reacted with an affinity-purified antibody raised against a synthetic peptide consisting of the 15 amino acids of the carboxy terminal of rat AQP2 [1, 2] diluted in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4) at a concentration of approximately 1 μg/ml for one hour at room temperature. The membrane was then washed three times in TBST and incubated with biotin-labeled antirabbit IgG (Vector, Burlingame, CA, USA) diluted at 200:1 in TBST for one hour at room temperature. After another three washes in TBST, the blots were visualized by ABC peroxidase mixture (Vector) diluted 500:1 in TBST and ECL chemiluminescence (Amersham) according to the manufacturer's instructions. Images of the bands were taken by an ECL Minicamera (Amersham).

Immunofluorescence

After 24 hours, the transfected cells grown on Biocoat chamber slides (Becton Dickinson, Bedford, MA, USA) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes, washed, and permeabilized with 0.1% Triton X-100 in PBS for one minute. Fixed cells were blocked with goat serum for one hour at room temperature. The cells were incubated with affinity-purified rat AQP2 antibody diluted at 300:1 in PBS for one hour at room temperature, and for double staining, anti-Grp78 antibody (Stressgen, Victoria, Canada) was added to the solution at a dilution of 200:1. After washing the slides with PBS three times, the cells were reacted with FITC-conjugated antirabbit IgG antibody (Cappel, Durham, NC, USA) in PBS for one hour, and for double staining, Cy3-conjugated antimouse IgG (Amersham) was added at a dilution of 200:1. Incubation of transfected cells with secondary antibody alone, anti-AQP2 antibody together with Cy3-conjugated antimouse IgG, or anti-Grp78 together with FITC-conjugated antirabbit IgG did not give significant staining (data not shown). The cells were mounted with Vectashield (Vector). Some cells were incubated with 30 nM vasopressin (Sigma, St. Louis, MO, USA) for 30 minutes before fixation. Transferrin-tetramethylrhodamine conjugate was purchased from Molecular Probes (Eugene, OR, USA) and was used at a concentration of 20 μg/ml in serum-free DMEM to promote endocytosis. Images were acquired on LSM510 (Carl Zeiss, Oberkochen, Germany)

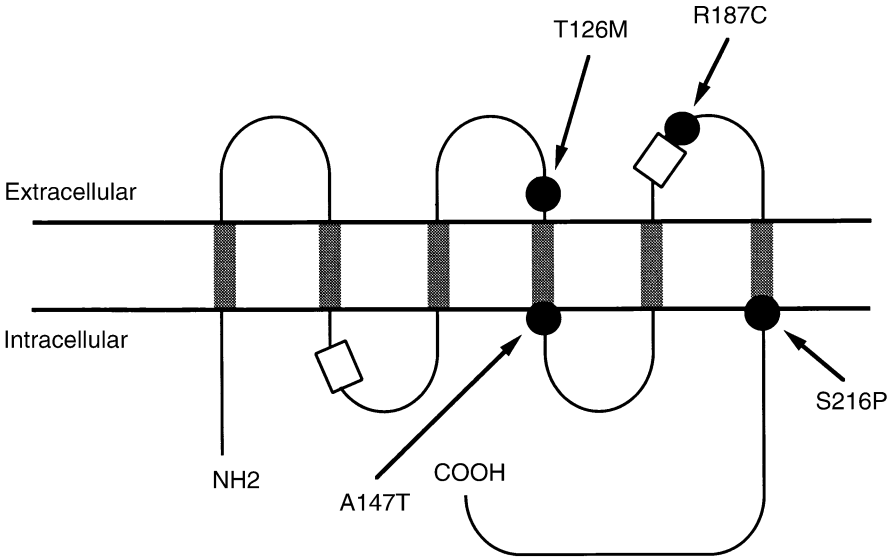

Fig. 1. Structure model of aquaporin-2 (AQP2). AQP2 is a 271-amino acid membrane integral protein that is predicted to have six transmembrane segments by homology to other members of the membrane intrinsic protein (MIP) family. NPA (asparagine, proline, allanine) domains highly conserved among aquaporins are shown with open boxes. The positions of the point mutations constructed in this study are also indicated with closed circles and arrows.

with appropriate software using a ×63 oil-immersion objective. Illumination was provided by a 488 nm argon laser for FITC and a 543 nm helium/neon laser for rhodamine/Cy3. Images for double-stained samples were collected synchronously using multiple channels. Emission filters used were LP 505 for samples stained with FITC alone, BP 505-530, and LP 560 for double-stained samples with FITC and Cy3/rhodamine.

Cell surface labeling and immunoprecipitation

The transfected cells were trypsinized, washed, and suspended in 1 mg/ml sulfo-NHS-LC-Biotin (Pierce) in biotinylation buffer and were incubated at 4°C for 45 minutes [18]. Half of the cells were incubated in 30 nM vasopressin (Sigma) for 30 minutes before suspension. Labeled cells were washed twice with PBS and were lyzed with Reporter Lysis Buffer (Promega) at 4°C for 15 minutes. Twenty microliters of protein A agarose (Oncogene Research Products, Cambridge, MA, USA) were added to preclean the lysate, and the lysate was then incubated at 4°C for one hour. The supernatant was collected and reacted with affinity-purified rat AQP2 antibody diluted at 500:1 at 4°C for one hour. AQP2 was immunoprecipitated by adding 20 μl of protein A agarose and rotating end-over-end overnight at 4°C. The samples were washed with the lysis buffer five times and suspended in 20 μl sodium dodecyl sulfate sample buffer. After denaturing at 70°C for 10 minutes, the samples were electrophoresed in a 10 to 20% gradient polyacrylamide gel and transferred to a nitrocellulose membrane as described earlier here. AQP2 was detected by ABC peroxidase mixture and ECL chemiluminescence methods, as described earlier in this article [15].

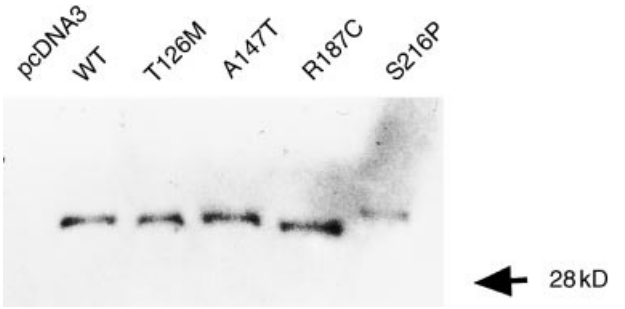

Fig. 2. Western blot of lysate of LLC-PK1 cells transfected with wild-type (WT) and AQP2 missense mutants. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted. The location of molecular mass standard of 28 kDa is indicated on the right.

Water permeability measurement

Water permeability measurement of transfected LLC-PK1 cells was performed as described previously [15]. Briefly, transfected cells were trypsinized, washed, and suspended in DMEM. Using a stopped-flow apparatus (SX18-MV; Applied Photophysics Ltd., Leatherhead, UK) equipped with a circulating water bath, the cell suspension and 600 mOsm mannitol in PBS were mixed abruptly giving a 300 mM inward osmotic gradient, and the change in cell volume was detected by monitoring the change in light scattering (466 nm wavelength at 90°). Light scattering intensity was fitted in a single exponential plot, and Pf was calculated by solving the following equation:

$$dV(t)/dt = Pf \times SAC \times vw \times (Osm_{in}/V(t) - Osm_{out})$$

where V (t) is the volume of the cell at time t, SAV is the surface area to volume ratio at t = 0 (6×10^{-5} cm^{-1}),

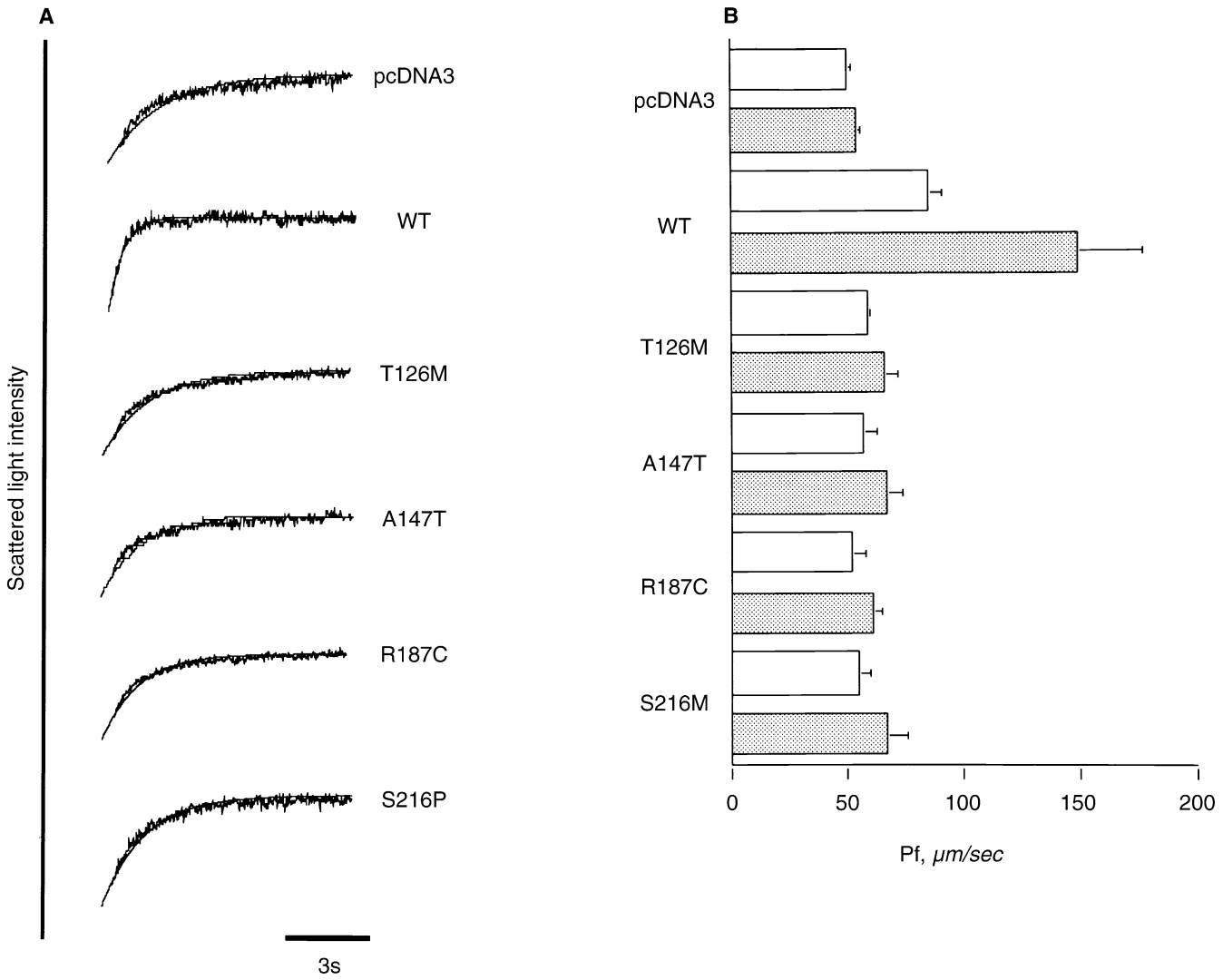

Fig. 3. (*A*) Representative traces of light scattering changes detected with a stopped-flow apparatus. Cells transfected with mock (pcDNA3), wild-type (WT), or AQP2 mutants were exposed to a 300 mOsm inward osmotic gradient by rapid mixing with 600 mM mannitol. These traces are from cells stimulated by incubation in 30 nM vasopressin at 37°C for 30 minutes. (*B*) The osmotic water permeability of transfected LLC-PK1 cells. Cells were incubated with (hatched bars) or without (open bars) 30 nM vasopressin for 30 minutes before measurement. Pf of WT-transfected cells increased significantly when incubated with vasopressin ($P < 0.05$, Student's *t*-test). Pf of AQP2 mutants was not significantly different from that of control and was not altered by vasopressin stimulation. Mean and SEM values are shown from five independent sets of measurements.

Vw is the molecular volume of water (18 cm^3/mol), Osm_{in} is the intracellular osmolarity, and Osm_{out} is the extracellular osmolarity. Some cells were incubated with 30 nM vasopressin for 30 minutes before measurements.

RESULTS

The expression of WT AQP2 and missense mutants T126M, A147T, R187C, and S216P (Fig. 1) in LLC-PK1 cells was confirmed by Western blot analysis of whole cell lysates of transfected cells. Immunoblots showed similar intense bands of approximately 29 kDa for WT, T126M, A147T, and R187C, indicating comparable expression of WT and these mutants (Fig. 2). Cells mock transfected with pcDNA3 expression vector produced no bands. The detected band of S216P was slightly fainter and apparently larger than WT, indicating instability of the mutant channel as previously reported [6] and difference in glycosylation. In preliminary lectin blot analysis, it was found that only S216P reacted with concanavalin A (data not shown), suggesting dominant high mannose glycosylation of S216P.

To examine whether NDI-related mutants fail to increase cell osmotic water permeability in LLC-PK1 cells in the same manner that they do in native kidney collecting duct cells, the osmotic water permeability (Pf) of LLC-PK1 cells expressing AQP2 and its mutants was determined by stopped-flow light-scattering micropho-

tometry. Transfected cells were exposed to a 300 mOsm inward osmotic gradient by rapid mixing, and the shrinkage of suspended cells was detected by the change in light scattering. Representative traces of light scattering are shown in Figure 3A, and Pf values of WT and mutant AQP2 are summarized in Figure 3B. Although WT-expressing cells showed a significant increase in water permeability after vasopressin stimulation, a significant increase in osmotic water permeability was not observed in LLC-PK1 cells transfected with the mutants even when stimulated with vasopressin. Our results indicated that LLC-PK1 cells are a suitable cell expression system as an experimental model to analyze the cell biology and molecular mechanisms of the pathogenesis of NDI caused by AQP2 mutations.

To determine subcellular localization and regulatory redistribution of AQP2 mutants, transfected LLC-PK1 cells were fixed and stained with affinity-purified rabbit antibody to rat AQP2. In approximately 70% of AQP2 WT-transfected cells, the expression of AQP2 was detected with bright immunofluorescence. Mock-transfected cells gave only background fluorescence (Fig. 4). Immunohistochemical localization of AQP2 WT and A147T at the basal condition was nearly identical to that of endocytosed transferrin, indicating that these proteins mainly reside in the endosome compartments (Figs. 4 and 5A) [19, 20]. In addition, the perinuclear dense staining observed in cells expressing WT and A147T (Fig. 5A, arrows) may represent the localization of WT and A147T in the perinuclear recycling vesicles also demonstrated in cells expressing the insulin-sensitive glucose transporter GLUT4 [21, 22]. Furthermore, when incubated with vasopressin, WT- and A147T-transfected cells showed a prominent basolateral distribution (Fig. 4, arrowheads) showing regulatory trafficking of WT and A147T to the surface membrane. Thus, it seemed that the missense mutation A147T did not disturb intracellular routing and vasopressin-regulated trafficking of the mutant in LLC-PK1 cells. On the contrary, mutants T126M, R187C, and S216P showed reticular staining colocalizing with an endoplasmic reticulum (ER) resided protein Grp78 (Figs. 4 and 5B) [23, 24], but they did not show any significant change in localization after vasopressin stimulation. This suggests that the missense mutations T126M, R187C, and S216P disrupted protein processing, intracellular routing, and regulatory trafficking of AQP2.

Cell-surface biotin labeling and immunoprecipitation were used to quantitate the cell surface expression of AQP2. Immunoprecipitants of transfected LLC-PK1 lysate showed that the cell-surface expression of WT and A147T increases significantly, whereas other mutants show little response to vasopressin stimulation (Fig. 6). These results further confirmed that the vasopressin-responsive trafficking of the mutant A147T is preserved.

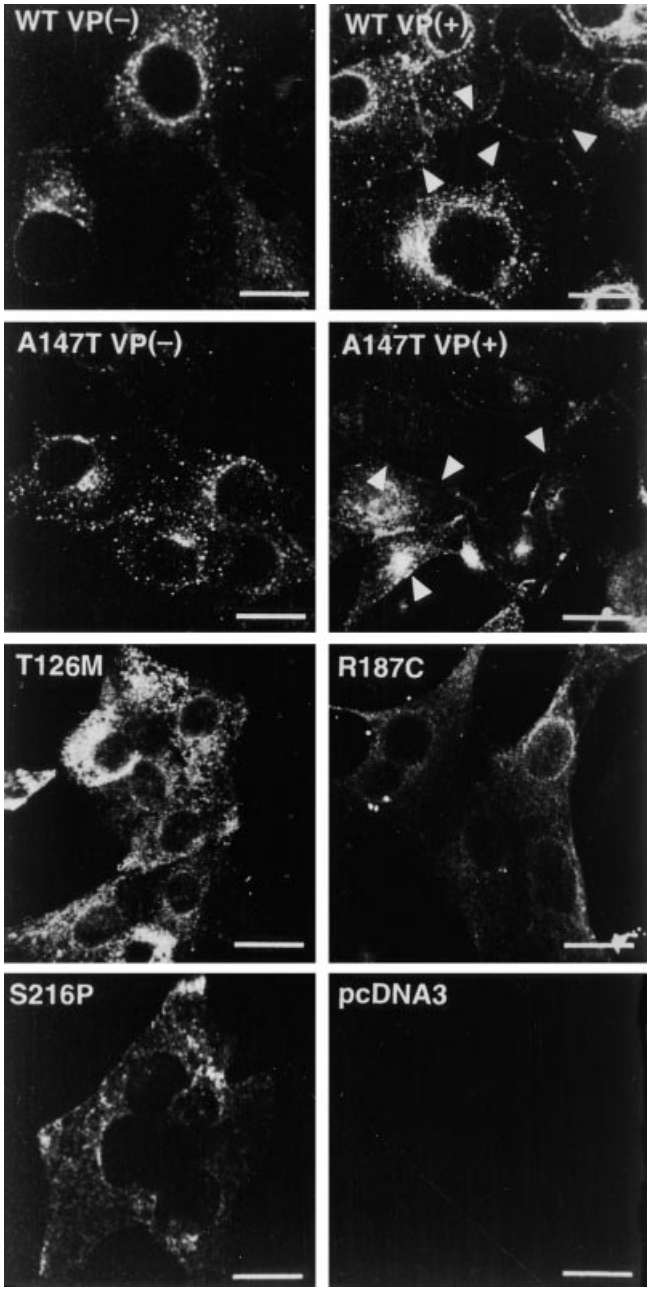

Fig. 4. Immunolocalization of AQP2 mutants in LLC-PK1 cells. Transfected LLC-PK1 cells were fixed with paraformaldehyde and immunostained with antibody to rat AQP2 and FITC-conjugated secondary antibody. Without vasopressin stimulation, stainings of most of LLC-PK1 cells expressing wild-type (WT) and A147T were compatible with perinuclear endosomal pattern. In contrast, the majority of T126M-, R187C-, and S216P-transfected cells showed an ER-like reticular staining pattern. Endosome versus endoplasmic reticulum staining pattern in AQP2-expressing cells were counted randomly in 10 different visual fields at ×630 as described [11]. Endosome-like perinuclear staining was seen in 70.7% of A147T- and 79.5% of WT-expressing cells, whereas ER-like reticular staining was seen in 79.4% of T126M-, 74.1% of R187C-, and 78.0% of S216P-expressing cells. Vasopressin-regulated redistribution of AQP2 was examined by incubating cells with 30 nM vasopressin at 37°C for 30 minutes [VP (+)]. Linear basolateral staining was noted in WT- and A147T-expressing cells (indicated by arrowheads), but stainings of cells expressing T126M, R187C, and S216P did not change significantly (data not shown; bars indicate 20 μm).

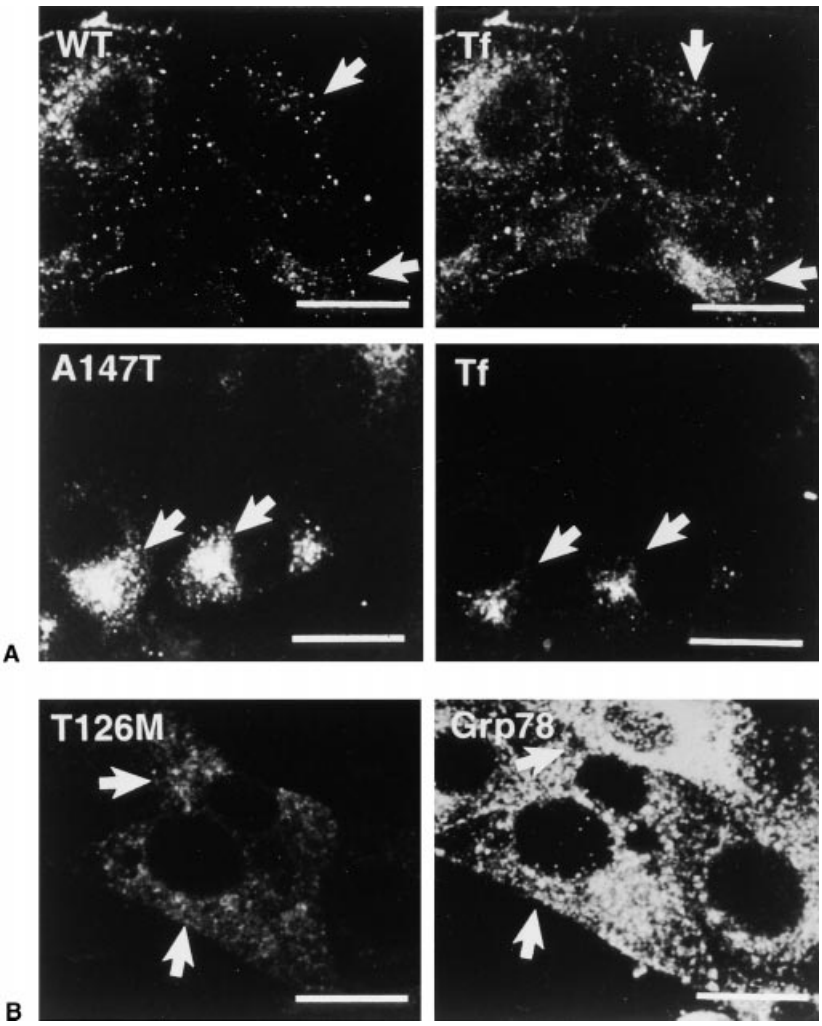

Fig. 5. Colocalization of wild-type (WT) and A147T with transferrin-rhodamine (Tf) and colocalization of T126M with Grp78. (*A*) Transfected LLC-PK1 cells were incubated with 20 mg transferrin-rhodamine for one hour at 37°C. Cells were washed with phosphate-buffered saline and fixed and stained with antibody to rat AQP2 and FITC-conjugated secondary antibody. Perinuclear dense stainings (shown by arrows) may represent the recycling endosome compartments. Intracellular localization of WT and A147T was nearly identical to that of rhodamine transferrin, showing distribution of WT and A147T in the endosomal compartments. (*B*) Transfected cells were fixed and double stained with antirat AQP2 antibody and anti-Grp78 antibody. FITC-conjugated and Cy3-conjugated secondary antibodies were used for immunodetection, respectively. The arrows indicate T126M-expressing cells stained with anti-Grp78. Note similar staining patterns for T126M and Grp78, indicating localization of T126M in the ER compartments. Stainings for R187C and S216P were similar to that for T126M (data not shown; bars indicate 20 μm).

DISCUSSION

Expression of four NDI-related mutations, T126M, A147T, R187C, and S216P, did not induce high water permeability of LLC-PK1 cell membrane even after vasopressin stimulation. Confocal immunohistochemistry revealed that WT and A147T resided in the recycling endosomal compartments in LLC-PK1 cells and that vasopressin stimulation translocated the channel proteins to the surface membrane. In contrast, mutants T126M, R187C, and S216P were shown to localize in the ER compartments, and vasopressin-regulated redistribution of these mutants was not observed. Thus, we conclude that A147T AQP2 is a nonfunctional water channel with intact regulatory trafficking and that the other three mutants have defects in routing to the surface membrane.

This study is the first to dissect the molecular characteristics of AQP2 mutants not modified with tag attachment in vasopressin-responsive kidney-derived epithelial cells. In previous reports, the function and routing of AQP2 mutants were examined in nonepithelial expression systems [5–11], or mutants were tagged with an artificial peptide for immunohistochemical detection [11, 13, 14]. In our examination, the behavior of the A147T mutant was similar to WT and different from that in previous reports [8, 11]. This discrepancy may be due to the differences in experimental conditions described earlier in this article. It is conceivable that routing of channel protein may be different in nonepithelial cells such as oocytes from native epithelial cells, that regulatory trafficking cannot be examined in these cells, and that tag modification may disturb the distribution of membrane proteins. It can be speculated that results with oocytes or nonepithelial cells may be misleading because the A147T mutant retains the responsiveness to vasopressin-regulated translocation or epithelial cell-specific trafficking that does not exist in these cells. Although mutational analyses on rat AQP2 may need to be carefully evaluated, our results are conceivably applicable to human NDI cases because the functions and structures of rat and human AQP2 are highly homologous (amino

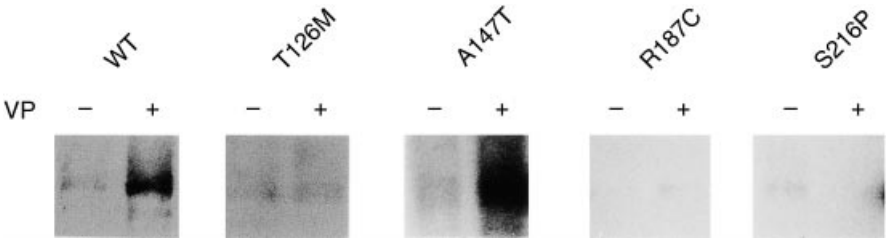

Fig. 6. Cell surface expression of wild type (WT) and AQP2 mutants in LLC-PK1 cells. WT- and mutant AQP2-transfected cells incubated with or without 30 nM vasopressin (VP +) at 37°C for 30 minutes before labeling with NHS-biotin. Biotin-labeled AQP2 was immunoprecipitated and visualized by ABC and ECL chemiluminescence methods. WT- and A147T-transfected cells show more dense bands (+) compared with nonstimulated cells (−), indicating an increase in cell surface expression.

acid sequence has 89.7% homology) [1, 5] and the analyzed residues are conserved.

LLC-PK1 cells showed basolateral insertion of AQP2 as opposed to *in vivo* insertion into the apical membrane. The reason for this is not clear but may be due to the limitation of culturing cells on flat glass slides or some intrinsic character of the LLC-PK1 cell line. The same expression pattern was seen with c-Myc–tagged AQP2 expressing LLC-PK1 cells established by Katsura et al, indicating that c-Myc tagging of AQP2 did not disturb the polarity of AQP2 expression [13]. Previous reports show that vasopressin regulation and phosphorylation recycling of AQP2 is intact in LLC-PK1 cells [13–15], which makes it a good tool for studying the machinery of cell surface expression of AQP2.

Together with the previous report that showed T125M and G175R expressed in oocytes failed to increase membrane water permeability [10], our finding that A147T is a nonfunctional water channel suggests that functional disruption of AQP2 by missense mutations accounts for the pathogenesis of some types of congenital NDI. As many cases of misrouting of AQP2 mutants have been reported [5–9, 11], it is inferred that the pathogenesis of NDI is heterogeneous. T125M mutation disrupts the N-glycosylation consensus sequence. Therefore, it was surprising that a defect in AQP2 trafficking was not observed. However, Baumgarten et al reported that glycosylation is not necessary for the translocation of AQP2 in MDCK cells [25], which raises questions on the role of glycosylation of AQP2. In addition, our observation is compatible with the previous analysis of AQP2 structure [26], in which the significance of the third and fourth hydrophilic loops was enhanced for the functional integrity of the aqueous pore. As A147 resides near the intracellular side of the fourth transmembrane segment, its mutation may interact with the assembly of the water pathway.

Immunolocalization indicated that T126M, R187C, and S216P have a defect in protein maturation, as suggested in previous examinations in oocytes [5–8]. Colocalization of these mutants with Grp78, an ER-residing protein [23, 24], suggested that some change in structure of the mutant AQP2 molecules may be detected and the proteins are retarded in the ER of LLC-PK1 cells. The ER is known to have a quality-control mechanism that prevents mutated, misfolded, or inappropriately complexed proteins from leaving it to the Golgi for further modifications [27–29]. These immature proteins are left in the ER and/or transported to lysosomes for degradation. Experiments with cystic fibrosis transmembrane conductance regulator (CFTR) show that some mutated channels cannot reach the cell surface because of defects in its processing. ΔF508, the most frequently found mutation of CFTR, is unable to mature to its fully glycosylated form, and this leads to intracellular retention of mutant CFTR protein [29–31].

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